

Research Article

Cite this article: Bastos TSA *et al.* (2020). Epidemiological and molecular identification of *Trypanosoma vivax* diagnosed in cattle during outbreaks in central Brazil. *Parasitology* **147**, 1313–1319. <https://doi.org/10.1017/S0031182020001006>

Received: 13 March 2020
Revised: 10 June 2020
Accepted: 11 June 2020
First published online: 22 June 2020



Key words:

Biting flies; epidemiology; iatrogenic route; molecular characterization; risk factors; trypanosomosis

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Epidemiological and molecular identification of *Trypanosoma vivax* diagnosed in cattle during outbreaks in central Brazil

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Abstract

Bovine trypanosomosis has been spreading in Brazil. In the present study, we evaluated the spatial distribution, prevalence and risk factors of this disease in the state of Goiás, Brazil, and performed both molecular and phylogenetical analyses of *Trypanosoma vivax*. A total of 4049 blood samples were collected from cattle for a period of 2 years. The parasitological diagnosis was performed using the Woo method and a questionnaire was administered to the farmers to document risk factors associated with the disease in the herd. Positive samples were DNA sequenced and compared to GenBank codes. The prevalence of *T. vivax* was 8.84%, occurring on 24 ranches only in dairy cattle and mainly in the central and southern portions of the state. The acquisition of new animals infected with *T. vivax* and the administration of exogenous oxytocin to cows using the same syringe and needle were the main associated factors ($P \leq 0.05$). After an outbreak, milk production decreased by 39.62%. The presence of biting flies (tabanids, *Haematobia irritans* and *Stomoxys calcitrans*) was not a risk factor ($P > 0.05$) for the occurrence of *T. vivax*. The epidemiological data demonstrate the importance of restricting the practice of auctions as well as eliminating the use of exogenous oxytocin in animals during milking. The samples tested by polymerase chain reaction were positive for *T. vivax* and were genetically homologous with *T. vivax* found in different states of Brazil and west Africa based on the 18S rRNA gene.

Introduction

Trypanosoma vivax causes trypanosomosis in cattle. This haemoprotozoan originating from Africa is believed to have been introduced in South America around the year 1830, with the transport of infected cattle from Senegal (Ventura *et al.*, 2001; Osório *et al.*, 2008). In sub-Saharan Africa, the transmission of this protozoa occurs biologically through the tsetse fly (*Glossina* spp.). In Central and South America, transmission occurs mechanically by biting flies (tabanids, *Stomoxys calcitrans* and *Haematobia irritans*) or by iatrogenic means (Cadioli *et al.*, 2012; Dagnachew and Bezie, 2015; Bastos *et al.*, 2017).

The first outbreak of trypanosomosis in Brazil occurred in the state of Pará in 1972 (Shaw and Lainson, 1972). From the year 2000 to 2017, occurrences have been reported in nearly 60% of the states of the country: Mato Grosso do Sul, Mato Grosso, Paraíba, Maranhão, Tocantins, Minas Gerais, São Paulo, Rio Grande do Sul, Pernambuco, Alagoas, Goiás, Sergipe, Piauí, Rio de Janeiro and Rio Grande do Norte (Silva *et al.*, 1996; Paiva *et al.*, 2000; Linhares *et al.*, 2006; Batista *et al.*, 2007; Carvalho *et al.*, 2008; Guerra *et al.*, 2008; Silva *et al.*, 2009; Cadioli *et al.*, 2012; Pimentel *et al.*, 2012; Andrade Neto *et al.* 2015; Costa *et al.*, 2016; Bastos *et al.*, 2017; Vieira *et al.*, 2017; Lopes *et al.*, 2018).

In the present study, we evaluated the spatial distribution, prevalence and risk factors of the acute occurrence of trypanosomosis in cattle in the state of Goiás, Brazil, and performed molecular analyses for the identification of *T. vivax* during an outbreak, with comparisons to previous findings based on the 18S rRNA gene.

Materials and methods

Ethics statement

This study received approval from the Animal Use Ethics Committee of the Federal University of Goiás, Brazil (certificate number: 032/15) and was conducted in compliance with the ethical principles governing animal experimentation of the Brazilian National Animal Experimentation Control Council (CONCEA).

Properties and animals for the study of *T. vivax*

The properties registered with the Agriculture and Livestock Defense Agency of the state of Goiás (AGRODEFESA-GO) that did not receive any medication with specific action against *T. vivax*, had cattle with acute problems suggestive of trypanosomiasis and reported recent animal deaths were selected for visits between May 2015 and May 2017. The owners were contacted by phone for visit arrangements.

Forty-two ranches were visited in 26 cities in the state of Goiás: Alexânia, Anápolis, Bonfinópolis, Buriti Alegre, Caldas Novas, Campo Alegre de Goiás, Corumbáiba, Cromínia, Edealina, Gameleira de Goiás, Goianápolis, Goianésia, Goiatuba, Guapó, Ipameri, Itaberaí, Itauçu, Jataí, Mairipotaba, Mambai, Morrinhos, Pontalina, Porteirão, Quirinópolis, Santa Bárbara de Goiás and Urutá. A total of 4049 blood samples were collected from cattle. Approximately 73% of the bovines evaluated were the Girolando breed (1/2 Holstein + 1/2 Gyr and 3/4 Holstein + 1/4 Gyr). The other breeds were Holstein (7/8 Holstein + 1/8 Gyr and 15/16 Holstein + 1/16 Gyr), Gyr, Jersey or crossbreed/Nelore.

Animal histories and blood collections were taken from at least 90% of the animals on each range. Owners in all regions of the state of Goiás were contacted, regardless of the type of ranch (dairy, beef or mixed). On each dairy farm, the history of daily milk production before and after the occurrence of *T. vivax* was investigated.

Parasitological diagnosis of *T. vivax* on ranches during the outbreak

The Woo method (Woo, 1970) was used to determine the presence of *T. vivax* in the blood samples. Approximately 4 mL of blood was collected from the caudal vein of each animal in a tube containing an anticoagulant (EDTA). Immediately after collection, the samples were homogenized and the blood was transferred to microhaematocrit tubes. Each tube was placed in a micro-centrifuge. After 5 min of centrifugation (13 000 RCF), the tube was examined under an optical microscope (magnification: 400×) for the study of *T. vivax* trypomastigotes.

Spatial distribution of *T. vivax* and risk factors

The state of Goiás is located in the midwestern region of Brazil and has an area of 340 106 km². To facilitate the interpretation of the spatial distribution of the registered cases, the data were grouped by mesoregion. The division of mesoregions (central, eastern, northern, northwestern and southern) as well as the division of the municipalities in which *T. vivax* was detected in cattle followed the division established by the Brazilian Institute of Geography and Statistics (IBGE, 2019).

Possible risk factors for cattle to acquire *T. vivax* in the state of Goiás were determined using a questionnaire administered at each property visited. The questionnaire addressed the following: type of ranch (dairy, beef or mixed); animal category presenting problem (lactating cows, dry cows, heifers, calves, bulls, steers, calves); whether animals had been purchased in the previous 90 days and the place of purchase; whether abortions occurred in this period; whether sick animals presented difficulty moving; whether a

Table 1. Association analysis between the mesoregions of the state of Goiás where *Trypanosoma vivax* was diagnosed in cattle

Mesoregions ^a	Total of animals	Mesoregion representativity (%) in relation to the total number of cattle evaluated		Protozoan		Prevalence (%)		Relative risk			Odds ratio				
		Positive	Negative	Value	95% CI	Value	95% CI	z statistic	Significance level	Value	95% CI	z statistic	Significance level		
East	589	2	587	0.34	0.13	0.81	1.00	1.00	-	-	-	-	-		
Central	2032	194	1838	9.55	8.27	10.82	28.17	7.00	112.89	4.70	30.98	7.67	125.14	4.82	<0.0001
South	1428	162	1266	11.34	9.70	12.99	33.41	8.31	134.29	4.94	37.56	30.12	46.82	32.22	<0.0001
-	4049	358	3691	8.84	7.97	9.72	-	-	-	-	-	-	-	-	-

No positive animals were detected in North and Northwest mesoregions of the State.

^aMesoregions with odds ratio and relative risk >1 (95% CI > 1) are more likely to contain cattle infected with *Trypanosoma vivax*

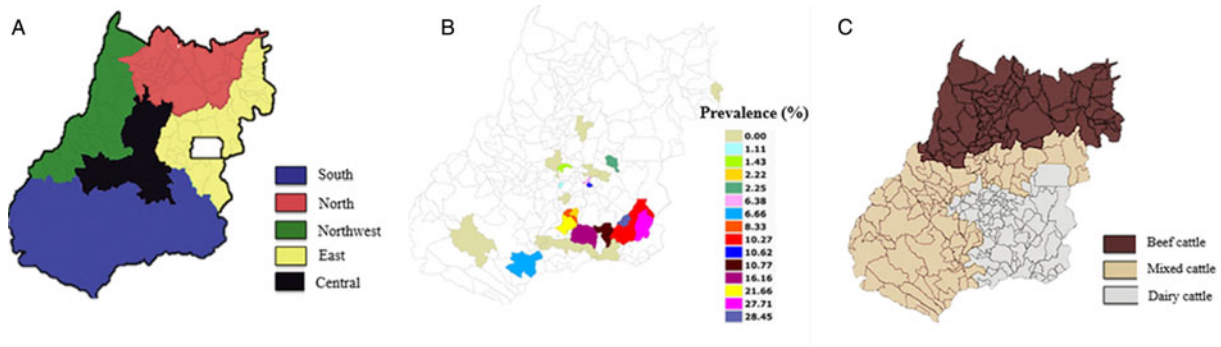


Fig. 1. Mesoregions of Goiás State, Brazil (A). Spatial distribution of *Trypanosoma vivax* in Goiás State, Brazil between May 2015 and May 2017 (B). Distribution of cattle exploitation in Goiás State, Brazil (C). Adapted from Rocha *et al.* (2009).

reduction in milk production had recently occurred; whether oxytocin was administered to lactating cows and whether the same syringe and needle were shared among different animals; whether haematophagous flies (such as *Tabanus*, *S. calcitrans* or *H. irritans*) were present on the property; and whether artificial insemination was employed on the ranch.

Molecular identification of *T. vivax* and phylogenetic analysis

Blood samples were sequenced (18S rRNA gene) and the phylogenetic analysis was performed for 20 of the 24 ranches visited on which the parasitological diagnosis confirmed animals positive for *T. vivax*: Alexânia, Bonfinópolis 01, Bonfinópolis 02, Caldas Novas, Goianópolis, Inhumas, Ipameri 01, Ipameri 02, Ipameri 03, Ipameri 04, Ipameri 05, Ipameri 06, Itauçu 01, Itauçu 02, Morrinhos, Pontalina 01, Pontalina 02, Quirinópolis, Urutaí 01, Urutaí 02 and Urutaí 03. The positive control was Ipameri 01 registered in GenBank (accession code MK392089). The samples from the four remaining ranches (Campo Alegre de Goiás, Cromínia, Mairipotaba and Santa Bárbara de Goiás) that had animals infected with *T. vivax* were insufficient to perform the analyses.

The detection and molecular characterization of *T. vivax* were performed as described by Vieira *et al.* (2017). Genomic DNA was extracted from 200 μ L of bovine blood using a commercial kit (Kasvi, Brazil) following the manufacturer's recommendations. Polymerase chain reaction (PCR) was performed using the primers 18STnF2 (5-CAACGATGACACCCATGAATTGGGGA-3) and 18STnR3 (5-TGCGCGACCAATAATTGCAATAC-3), which amplified a fragment of 659 bp of the 18S rRNA gene of *T. vivax*. The identity of the DNA sequences was determined by comparisons with sequences available in GenBank using BLASTn. A phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA).

Statistical analysis

The data on the total number of animals diagnosed with *T. vivax* in the mesoregions and municipalities of the state of Goiás, Brazil, were used to calculate the prevalence. Subsequently, prevalence data were arranged in ascending order both for mesoregions and municipalities, setting the odds ratio (OR) equal to 1 for the lowest observed prevalence, then calculating the other ORs in relation to this. The Z test was used to determine the significance, considering a 95% significance level ($P \leq 0.05$).

Regression analysis was employed to determine associations between the prevalence [dichotomized by the median (zero for values below and one for values above the median)] of *T. vivax* and all the epidemiological variables described above. With these data, a simple binary logistic regression analysis was applied for all epidemiological variables and only those with a P value \leq

0.20 in the univariate analysis were selected for the multivariate logistic regression analysis. The strength of the associations between the dependent and independent variables was estimated using ORs derived from the logistic regression estimates. Variables with a $P \leq 0.05$ in the multivariate analysis were considered to be significantly associated with the outcome. A correlation analysis was also performed on the prevalence of *T. vivax* and epidemiological data collected during the visits. For such, Spearman's correlation coefficients were calculated. All statistical procedures were conducted using Epi Info, version 7.1.5.2.

Results

Prevalence of *T. vivax* in the state of Goiás during outbreaks

Trypanosoma vivax was found on 24 ranches distributed among 14 municipalities only in Girolando dairy cattle and only in lactating and/or dry cows. Among a total of 4049 blood samples analysed using the Woo method, 358 (8.84%; CI 95% 7.97–9.72) were positive for the acute form of trypanosomosis, as shown in Table 1 and Figs. 1A–C.

Prevalence rates, ORs and RRs by mesoregion in the state of Goiás are displayed in Table 2 and Fig. 1B. No animals positive for *T. vivax* were found in 12 of the 26 municipalities of origin: Anápolis, Edealina, Buriti Alegre, Corumbáiba, Gameleira de Goiás, Goianésia, Goiatuba, Guapó, Itaberaí, Jataí, Mambai and Porteirão. On four of the properties where *T. vivax* was not found (located in the municipalities of Mambai, Buriti Alegre, Jataí and Quirinópolis), the blood analysis revealed high parasitism by *Anaplasma marginale* in cows, which was probably the cause of some deaths reported by the owners when answering the questionnaire.

Risk factors associated with epidemiological variables

Among the epidemiological variables submitted to logistic regression analysis, the type of animal utilization (beef, dairy or mixed), animal category (lactating cows and others), purchase of animals in the previous 90 days, a reduction in milk production and the use of oxytocin in lactating cows sharing the same syringe and needle were significantly associated ($P \geq 0.05$) with the occurrence of *T. vivax* in cattle in the state of Goiás, Brazil (Table 3).

Specifically, significant positive correlations ($P \leq 0.05$) were found between the prevalence of *T. vivax* and animal category ($\rho = 0.78$), type of cattle ($\rho = 0.68$), purchase of animals in the previous 90 days ($\rho = 0.38$), place of purchase of these animals ($\rho = 0.87$) and use of oxytocin in cows during milking sharing the same syringe and needle ($\rho = 0.69$). The purchase of animals in the previous 90 days also had a significant positive correlation ($P \leq 0.05$) with the occurrence of abortions ($\rho = 0.69$), locomotion

Table 2. Association analysis between the municipalities of the state of Goiás, regarding the prevalence of *Trypanosoma vivax* diagnosed in cattle

Municipals ^a	Mesoregion	Total of animals	Municipals representativity (%) in relation to the total number of cattle evaluated	Protozoan		Prevalence (%)			Relative risk				Odds ratio								
				Positive	Negative	Value	95% CI		Value	95% CI	z statistic	Significance level	Value	95% CI	z statistic	Significance level					
Anápolis	Central	110	2.72	0	110	0.00															
Edealina	South	25	0.62	0	25	0.00															
Buriti Alegre	Central	27	0.67	0	27	0.00															
Corumbaíba	South	25	0.62	0	25	0.00															
Gameleira de Goiás	South	40	0.99	0	40	0.00															
Goianésia	Central	10	0.25	0	10	0.00															
Goiatuba	South	50	1.23	0	50	0.00															
Guapó	Central	20	0.49	0	20	0.00															
Itaberaí	Central	25	0.62	0	25	0.00															
Jataí	South	120	2.96	0	120	0.00															
Mambai	East	500	12.35	0	500	0.00															
Porteirão	South	20	0.49	0	20	0.00															
Santa Bárbara de Goiás	Central	90	2.22	1	89	1.11	0.00	-	3.28	1.00					1.00						
Itaçu	Central	140	3.46	2	138	1.43	0.00	-	3.39	1.29	0.12	-	13.97	0.21	0.8364	1.29	0.12	-	14.44	0.21	0.8364
Cromínia	South	45	1.11	1	44	2.22	0.00	-	6.53	2.00	0.13	-	31.24	0.49	0.6211	2.02	0.12	-	33.11	0.49	0.6213
Alexânia	East	89	2.20	2	87	2.25	0.00	-	5.33	2.02	0.19	-	21.91	0.58	0.5623	2.05	0.18	-	22.98	0.58	0.5618
Goianópolis	Central	47	1.16	3	44	6.38	0.00	-	13.37	5.74	0.61	-	53.72	1.53	0.1253	6.07	0.61	-	60.03	1.54	0.1231
Mairipotaba	South	12	0.30	1	11	8.33	0.00	-	23.97	7.50	0.50	-	112.23	1.46	0.1444	8.09	0.47	-	138.73	1.44	0.1493
Quirinópolis	South	20	0.49	2	18	10.00	0.00	-	23.15	9.00	0.86	-	94.47	1.83	0.0670	9.89	0.85	-	114.98	1.83	0.0672
Ipameri	South	584	14.42	60	524	10.27	7.81	-	12.74	9.25	1.30	-	65.89	2.22	0.0264	10.19	1.39	-	74.48	2.29	0.0222
Bonfinópolis	Central	1440	35.56	153	1287	10.63	9.03	-	12.22	9.56	1.35	-	67.54	2.26	0.0236	10.58	1.46	-	76.49	2.34	0.0194
Caldas Novas	South	65	1.61	7	58	10.77	3.23	-	18.31	9.69	1.22	-	76.88	2.15	0.0316	10.74	1.29	-	89.60	2.19	0.0283
Morrinhos	South	99	2.45	16	83	16.16	8.91	-	23.41	14.55	1.97	-	107.48	2.62	0.0087	17.16	2.23	-	132.25	2.73	0.0064
Pontalina	South	240	5.93	52	188	21.67	16.45	-	26.88	19.50	2.74	-	138.97	2.96	0.0030	24.62	3.35	-	180.95	3.15	0.0016
Campo Alegre de Goiás	South	83	2.05	23	60	27.71	18.08	-	37.34	24.94	3.44	-	180.60	3.18	0.0015	34.12	4.49	-	259.44	3.41	0.0006
Urutaí	Central	123	3.04	35	88	28.46	20.48	-	36.43	25.61	3.57	-	183.48	3.23	0.0012	35.40	4.75	-	264.06	3.48	0.0005
-	-	4049	100.00	358	3691	8.84	7.97	-	9.72	-	-	-	-	-	-	-	-	-	-	-	-

^aMunicipals with odds ratio and relative risk >1 (95% CI >1) are more likely to contain cattle infected with *Trypanosoma vivax*.

type of cattle, the purchase of animals and use of oxytocin with the same syringe and needle in cows during milking. These aspects explain the rapid dissemination of this protozoan parasite among dairy cattle in this study.

The prevalence of *T. vivax* in cattle is causally related to the sensitivity of the diagnostic method employed. In a comparative study of indirect immunofluorescence, conventional PCR (cPCR) and the Woo method, cPCR proved the most sensitive for detecting low parasitaemia (Alves *et al.*, 2017; Rabelo *et al.*, 2017; Bastos *et al.*, 2020). However, the Woo method can be used to identify animals during the acute phase of infection in outbreaks of trypanosomosis, which justifies the use of this method in the present investigation, the aim of which was to diagnose acute cases of trypanosomosis in cattle.

Over the past 16 years, *T. vivax* has spread rapidly across Brazil (Linhares *et al.*, 2006; Batista *et al.*, 2007; Carvalho *et al.*, 2008; Guerra *et al.*, 2008; Silva *et al.*, 2009; Cadioli *et al.*, 2012; Pimentel *et al.*, 2012; Andrade Neto e André, 2015; Costa *et al.*, 2016; Bastos *et al.*, 2017; Vieira *et al.*, 2017; Lopes *et al.*, 2018). In 11 of the publications cited, outbreaks occurred in Girolando dairy cattle; 10 reported the introduction of new animals to the herd and seven reported the use of oxytocin and/or vaccines as the predisposing factor for the occurrence of trypanosomosis in the respective herds. The sale of these animals occurs collectively at auctions, which directly contributes to the dissemination of this protozoan to other properties. The use of intravenous oxytocin in lactating cows is a common management practice to increase the milking speed and reduce the time spent milking on properties with Girolando dairy cows (Araújo *et al.*, 2012). This practice is performed with the same needle and syringe on several animals, which contributes to the spread of diseases in the herd. Thus, in view of the results obtained in the present investigation and previous studies, we can state that the acquisition of new animals with *T. vivax* and the administration of exogenous oxytocin to cows during milking using the same syringe and needle are the main causes of the dissemination of trypanosomosis in the state of Goiás and other regions of Brazil.

The 39.62% reduction in average daily milk production found on properties with the presence of *T. vivax* may be explained by the persistent fever that the acute phase of this disease causes in cattle with high parasitaemia ($\geq 2 \times 10^6$). It is well known that sudden, persistent fever results in anorexia in animals (Cavalcante, 2000; Peixoto *et al.*, 2011). Moreover, other researchers describe a positive correlation with hyperthermia in cattle during the acute phase of trypanosomosis, which lends support to the inference offered here (Schenk *et al.*, 2001; Desquesnes, 2004; Almeida *et al.*, 2010; Dagnachew and Bezie, 2015).

The presence of tabanid flies, *S. calcitrans* or *H. irritans*, was not correlated ($P > 0.05$) with infection on the 24 properties in which *T. vivax* outbreaks were diagnosed. However, researchers have reported that animal–animal transmission in other regions of the country could be related to the presence of these mechanical vectors (Otte and Abuabara, 1991; Silva *et al.*, 1996; Batista *et al.*, 2012; Cadioli *et al.*, 2012; Batista *et al.*, 2018). The epidemiological data demonstrate the importance of restricting the practice of collective sales events (auctions) as well as eliminating the use of exogenous oxytocin in animals during the milking process to avoid serious problems for cattle breeders in Brazil. Therefore, further studies evaluating the dissemination capacity of *T. vivax* transmission by haematophagous flies and studies evaluating the propagation capacity of iatrogenic pathways of *T. vivax* are needed.

The phylogenetical analysis proved that the *T. vivax* found in Goiás, Brazil, was homologous to others from the same country and west Africa, but different from *T. vivax* from east Africa. These results are in agreement with data in the literature stating

that *T. vivax* in Brazil was originally from Africa (probably west Africa) (Osório *et al.*, 2008; Cortez *et al.*, 2006; Rodrigues *et al.*, 2008; Pimentel *et al.*, 2012; Vieira *et al.*, 2017).

Conclusion

The prevalence of trypanosomosis in the state of Goiás, Brazil, during the acute phase of the disease was 8.84%. The main risk factors for the dissemination of *T. vivax* in the herds were the acquisition of new animals infected with this protozoan and the administration of oxytocin in cows using the same syringe and needle among several animals. In contrast, the presence of tabanid flies, *S. calcitrans* and *H. irritans*, was not a risk factor for the occurrence of this haemoparasite. The *T. vivax* identified in the present study were genetically homologous to each other as well as others found in Brazil and west Africa.

Financial support. This work was supported by Fundo para o Desenvolvimento da Agropecuária em Goiás (FUNDEPEC-GOÍAS) (agreement signed on 5 May 2016) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – financial code 001.

Ethical standards. This study received approval from the Animal Use Ethics Committee of the Federal University of Goiás, Brazil (certificate number: 032/15) and was conducted in compliance with the ethical principles governing animal experimentation of the Brazilian National Animal Experimentation Control Council (CONCEA).

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